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Identification of Novel *Listeria monocytogenes* Secreted Virulence Factors following Mutational Activation of the Central Virulence Regulator, PrfA▽†

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Upon bacterial entry into the cytosol of infected mammalian host cells, the central virulence regulator PrfA of *Listeria monocytogenes* becomes activated and induces the expression of numerous factors which contribute to bacterial pathogenesis. The mechanism or signal by which PrfA becomes activated during the course of infection has not yet been determined; however, several amino acid substitutions within PrfA (known as PrfA* mutations) that appear to lock the protein into a constitutively activated state have been identified. In this study, the PrfA activation statuses of several *L. monocytogenes* mutant strains were subjected to direct isogenic comparison and the mutant with the highest activity, the *prfA*(*L140F*) mutant, was identified. The *prfA*(*L140F*) strain was subsequently used as a tool to identify gene products secreted as a result of PrfA activation. By use of two-dimensional gel electrophoresis followed by liquid chromatography-electrospray ionization–tandem mass spectroscopy analyses, 15 proteins were identified as up-regulated in the *prfA*(*L140F*) secretome, while the secretion of two proteins was found to be reduced. Although some of the proteins identified were known to be subject to direct regulation by PrfA, the majority have not previously been associated with PrfA regulation and their expression or secretion may be influenced indirectly by a PrfA-dependent regulatory pathway. Plasmid insertion inactivation of the genes encoding four novel secreted products indicated that three of the four have significant roles in *L. monocytogenes* virulence. The use of mutationally activated *prfA* alleles therefore provides a useful approach towards identifying gene products that contribute to *L. monocytogenes* pathogenesis.

Listeria monocytogenes is a gram-positive facultative intracellular bacterial pathogen capable of causing severe disease in humans following ingestion of contaminated food products (66). As an environmental pathogen, *L. monocytogenes* has several disparate lifestyles, which include life as a saprophyte in soil, water, and contaminated silage and life inside mammalian hosts (28). While little about patterns of bacterial gene expression in natural environments outside of host cells is known, *L. monocytogenes* gene expression profiles have been shown to shift dramatically when the bacterium transitions from planktonic growth in broth culture to the cytosol of infected mammalian host cells (10, 37). Many of the bacterial factors required for *L. monocytogenes* pathogenesis are regulated by a transcriptional regulator known as PrfA (47).

PrfA-dependent gene products play critical roles in every major step of bacterial pathogenesis, from host cell internalization of the bacteria and phagosomal escape to spread of the bacteria to adjacent cells (64, 66). *L. monocytogenes* induces its uptake into cells by using a variety of bacterial cell surface-associated molecules, including a family of PrfA-dependent internalin gene products (11, 17, 23). Once internalized, *L. monocytogenes* escapes from the intracellular vacuole through expression of two PrfA-dependent genes: *hly*, encoding the pore-forming toxin listeriolysin O (LLO) (12, 25, 39, 50), and *plcA*, encoding a phosphatidylinositol-specific phospholipase C (8). Upon entry into the cytosol, *L. monocytogenes* produces ActA, a PrfA-dependent gene product that directs host cell actin polymerization and enables bacterial spread into nearby host cells (40, 60). *L. monocytogenes* then escapes from the double membrane vacuole of the newly infected cell through the expression of *plcA*, *hly*, and an additional PrfA-dependent phospholipase encoded by *plcB* (59, 65). PrfA thus plays a critical role in *L. monocytogenes* pathogenesis by regulating the expression of genes required for multiple aspects of host infection (47).

The regulation of *prfA* expression and protein activity is quite complex and includes transcriptional (22), posttranscriptional (33), and posttranslational (52) mechanisms. While each regulatory mechanism is important for proper modulation of *prfA* expression and activity, the posttranslational regulation of PrfA is perhaps the least well understood. PrfA activation appears to require some form of protein modification or cofactor binding, and although the nature of the signal that mediates PrfA activation is not yet known, insight into the functional consequences of PrfA activation has been obtained through the isolation and characterization of PrfA mutants that lock the protein into a constitutively active state (referred to as *prfA** mutants) (52, 57, 67, 72). When grown in vitro, *prfA** mutants appear to mimic the natural activation of PrfA that occurs in *L. monocytogenes* within the cytosol of host cells where PrfA-dependent gene expression is highly up-regulated.

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TABLE 1. *L. monocytogenes* and *E. coli* strains used in this study

Strain	Description	Associated designation	Reference(s) or source
DH5α	*E. coli* strain for constructing recombinant plasmids		
SM10	*E. coli* strain as conjugation donor for pPL2 plasmid		
NF-L100	10403S wild type		3, 18
DP-L2161	10403S Δ*hly*	*hly*	36
DP-L3903	10403S with Tn*917* insertion		1
NF-L1003	NF-L890 (Δ*prfA*) with *actA-gus-plcB* transcriptional fusion		72
NF-E1019	*E. coli* DH5α with pPL2-P*plcA-prfA*		72
prfA mutants			
NF-L1009	NF-L1003 tRNA^{Arg}::pPL2 (Δ*prfA* plus pPL2*i*)[a]	Δ*prfA*	72
NF-L1011	NF-L1003 tRNA^{Arg}::pPL2-*prfA*(*L140F*) (Δ*prfA* plus L140F*i*)	*prfA*(*L140F*)	72
NF-L1041	NF-L1003 tRNA^{Arg}::pPL2-*prfA476* (Δ*prfA* plus WT*i*)	Wild-type *prfA*	72
NF-L1185	NF-L1003 tRNA^{Arg}::pPL2-*prfA*(*E77K*) (Δ*prfA* plus E77K*i*)	*prfA*(*E77K*)	This work
NF-L1186	NF-L1003 tRNA^{Arg}::pPL2-*prfA*(*G155S*) (Δ*prfA* plus G155S*i*)	*prfA*(*G155S*)	This work
NF-L1226	NF-L1003 tRNA^{Arg}::pPL2-*prfA*(*G145S*) (Δ*prfA* plus G145S*i*)	*prfA*(*G145S*)	This work
Insertion mutants			
NF-L1239	NF-L100 with pKSV7 single-crossover insertion within *actA*	*actA*i[b]	This work
NF-L1240	NF-L100 with pKSV7 single-crossover insertion within lmo0135	lmo0135i	This work
NF-L1241	NF-L100 with pKSV7 single-crossover insertion within *tcsA*	*tcsA*i	This work
NF-L1242	NF-L100 with pKSV7 single-crossover insertion within lmo2219	lmo2219i	This work
NF-L1243	NF-L100 with pKSV7 single-crossover insertion within lmo2417	lmo2417i	This work
NF-L1245	NF-L100 with pKSV7 single-crossover insertion near but not in lmo2219	Silent pKSV7 insertion	This work
Δ*flaA* mutant			
NF-L1246	NF-L965 tRNA^{Arg}::pPL2 (Δ*flaA* plus pPL2*i*)	Δ*flaA*	This work

[a] *i*, pPL2 plasmid insertion.
[b] i, pKSV7 plasmid insertion in designated ORF.

Posttranslation activation of PrfA is required for *L. monocytogenes* virulence, as mutations in *prfA* that prevent activation result in severe attenuation in murine infection models (M. D. Miner, G. C. Port, J. C. Chang, H. G. A. Bouwer, and N. E. Freitag, submitted for publication).

As PrfA is a key regulator of *L. monocytogenes* virulence, we sought to identify the spectrum of gene products both directly and indirectly regulated by PrfA in order to uncover additional bacterial factors that contribute to *L. monocytogenes* pathogenesis. As part of this approach, levels of PrfA-dependent gene expression were directly compared between several *prfA** mutant isogenic strains, thereby revealing the existence of two classes of *prfA** mutants: moderately activated *prfA** mutants [*prfA*(*E77K*) and *prfA*(*G155S*) mutants] and highly activated *prfA** mutants [*prfA*(*G145S*) and *prfA*(*L140F*) mutants]. Profiles of secreted proteins derived from the most active *prfA** mutant, the *prfA*(*L140F*) mutant, were then compared to profiles of secreted proteins isolated from wild-type as well as Δ*prfA* mutant strains. Fifteen proteins were found to be upregulated as a result of high-level PrfA activation, whereas two proteins were absent from the PrfA L140F secretome in comparison to wild-type and Δ*prfA* mutant strains. As anticipated, novel PrfA activation-dependent gene products which were found to contribute to *L. monocytogenes* pathogenesis were identified.

MATERIALS AND METHODS

Bacterial strains and plasmids. *L. monocytogenes* and *Escherichia coli* strains used in this study are listed in Table 1. All *L. monocytogenes* and *E. coli* strains were grown at 37°C in brain heart infusion (BHI) media (Difco Laboratories, Detroit, MI) and Luria broth (LB) (Invitrogen Corp., Grand Island, NY), respectively. For selection of pPL2 or its recombinant plasmids, chloramphenicol was used at 25 μg/ml for *E. coli* and at 5 to 10 μg/ml for *L. monocytogenes*. Streptomycin at 200 μg/ml was used to select for *L. monocytogenes* following bacterial conjugation and for the elucidation of bacterial CFU from the organs of infected mice (10403S is resistant to streptomycin).

Construction of *L. monocytogenes* recombinant strains. *L. monocytogenes* strains NF-L1009, NF-L1011, and NF-L1041 (72) are previously described derivatives of the 10403S parent strain. Briefly, all strains contain an in-frame *prfA* deletion as well as a transcriptional fusion of the reporter gene *gus* to *actA* within the *L. monocytogenes* chromosome. In addition, each strain contains the integrated plasmid vector pPL2 as empty vector (NF-L1009), with a wild-type copy of *prfA* (pPL2 *prfA*-WT [NF-L1041]), or with a *prfA** allele [pPL2-*prfA*(*L140F*) (NF-L1011)]. Isogenic strains containing additional *prfA** mutations were constructed as follows. Point mutations were introduced into pPL2 *prfA*-WT from pNF-E1019 by use of a QuikChange site-directed mutagenesis kit (Stratagene) with the oligonucleotides listed in Table S1 in the supplemental material according to the manufacturer's protocol to generate the *prfA*(*E77K*), *prfA*(*G155S*), and *prfA*(*G145S*) alleles. Plasmids containing the desired mutations within *prfA* in pPL2 were verified by sequencing. The resulting strains NF-L1185 [*prfA*(*E77K*)], NF-L1186 [*prfA*(*G155S*)], and NF-L1226 [*prfA*(*G145S*)] each contain a single copy of the integration vector pPL2 with the designated *prfA* allele inserted into the *L. monocytogenes* chromosome.

NF-L1246 (wild-type chromosomal *prfA*) was derived from nonmotile NF-L965 (Δ*flaA*) (57) conjugated with pPL2 vector in order to confer chloramphenicol resistance and to enable direct comparison of swimming motility with that of pPL2-complemented *prfA** strains.

Measurement of GUS activity. β-Glucuronidase (GUS) activity was measured as previously described (56, 57, 72), with some minor changes. Briefly, overnight cultures grown in BHI containing 5 μg/ml chloramphenicol (BHI cam^5) were diluted 1:50 and grown with shaking at 37°C for 8 h. The optical density at 600 nm (OD_{600}) was measured, and two 500-μl culture aliquots were collected hourly for all strains except for the *prfA*(*L140F*) (NF-L1011) and *prfA*(*G145S*) (NF-L1226) mutant strains, for which two 50-μl aliquots were collected (reflective of the increased GUS activity present in these two highly PrfA activated strains). Bacterial cells were recovered by centrifugation at 16,000 × *g* for 5 min, the supernatants were removed, and the bacterial pellets were frozen at −80°C for further analysis. For enzymatic assays, bacterial pellets were quickly thawed and

resuspended in 100 μl or 1 ml ABT buffer (0.1 M potassium phosphate, pH 7.0, 0.1 M NaCl, 0.1% Triton). GUS activity was measured as described by Youngman (73), with the substitution of 4-methylumbelliferyl-β-D-glucuronide in place of 4-methylumbilliferyl-β-D-galactoside (Sigma Chemical Co., St. Louis, MO). Data were derived from duplicate samples taken from three independent experiments.

Isolation of bacterial secreted proteins. For the isolation of secreted proteins, overnight cultures were diluted 1:20 into a final volume of 200 ml of prewarmed BHI cam[5] and grown at 37°C for approximately 3 h to mid-log phase (OD_{600} of ~0.6) with shaking. Culture OD_{600} was determined by using a spectrophotometer (UV-1201 UV-VIS spectrophotometer; Shimadzu Scientific Instruments, Inc., Columbia, MD).

Secreted proteins were isolated as previously described by Lenz and Portnoy (43), with minor modifications. Briefly, bacteria in 200 ml of mid-log-phase cell culture were pelleted by centrifugation at 20,000 × *g* for 5 min at 4°C and 160 ml of the resulting supernatants was collected. Trichloroacetic acid (TCA) was added to the supernatants to reach a final concentration of 5% TCA. Proteins were TCA precipitated on ice for 30 min. The supernatants were then centrifuged at 10,500 × *g* for 10 min at 4°C, the supernatants were discarded, and the resulting protein pellets were air dried. The TCA-precipitated protein pellets were washed with 4 ml of ice-cold acetone and centrifuged at 10,500 × *g* for 10 min at 4°C, decanted, and air dried again. The final pellets were resuspended in 400 μl of sodium dodecyl sulfate (SDS) boiling buffer containing 5% SDS, 10% glycerol, and 60 mM Tris, pH 6.8. Samples were boiled for 5 min before being frozen at −20°C until further analysis.

Protein concentrations were determined by bicinchoninic acid (BCA) analysis according to the manufacturer's protocol (Pierce, Rockford, IL). The absence of contaminating cytosolic proteins in the secreted protein samples was verified by Western analysis for the absence of PrfA protein in the secreted protein samples (data not shown).

Western analyses. Recombinant LLO and rabbit polyclonal LLO antiserum were obtained from Diatheva, Italy. IRDye 680 goat anti-rabbit immunoglobulin G fluorescently labeled secondary antibody was obtained from Li-Cor Biosciences, Lincoln, NE. For Western analysis, 45.5 μg of secreted proteins from each strain was separated on NuPage Novex 10% bis-Tris gels and run according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). The proteins were then transferred onto nitrocellulose membranes at 30 V for 60 min. Membranes were processed and proteins were visualized using a Li-Cor Odyssey imager according to the manufacturer's protocol, with Odyssey blocking buffer (Li-Cor, Lincoln, NE). Primary antiserum was diluted 1:1,000, while secondary antibody was diluted 1:20,000. Proteins were detected on the Odyssey imager by scanning in the 680-nm wavelength and quantified by comparison with 8 ng of loaded recombinant LLO protein using Odyssey infrared imaging system application software, version 2.1 (Li-Cor, Lincoln, NE). Secreted proteins were isolated and analyzed from three independent experiments.

2-DE of protein samples. Two-dimensional gel electrophoresis (2-DE) was performed by Kendrick Labs, Inc. (Madison, WI), according to the method of O'Farrell (48). Briefly, 5% β-mercaptoethanol was added to the protein samples and shipped overnight on dry ice to Kendrick Labs. Protein samples from each strain were loaded in equal concentrations based upon BCA analysis results, up to a maximum of 50 μl/gel (resulting in 250 to 500 μg/gel depending on the sample set). Isoelectric focusing (IEF) was carried out in glass tubes (inner diameter, 2.0 mm) by using 2.0% pH 4 to 8 ampholines (Gallard-Schlesinger Industries, Inc., Garden City, NY) for 9,600 V · h (IEF tube gel separation using carrier ampholines allows SDS-solubilized samples to be analyzed by 2-DE). One microgram of an IEF internal standard, tropomyosin (M_r 33,000, pI 5.2), was added to the samples. After IEF, the tube gels were equilibrated for 10 min in buffer O (10% glycerol, 2.3% SDS, 0.0625 M Tris, pH 6.8) and sealed to the top of a stacking gel that overlays 10% acrylamide slab gels (0.75 mm thick). The SDS slab gel electrophoresis was carried out for 4.5 h at 12.5 mA/gel. The following proteins were added as molecular weight standards to the agarose that sealed the tube gel to the slab gel: myosin (M_r 220,000), phosphorylase A (M_r 94,000), catalase (M_r 60,000), actin (M_r 43,000), carbonic anhydrase (M_r 29,000), and lysozyme (M_r 14,000) (Sigma Chemical Co., St. Louis, MO). Following electrophoresis, the gels were stained with Coomassie brilliant blue R-250 and dried between two sheets of cellophane. Samples were prepared from three independent experiments, and duplicate gels were run for each sample. 2-DE gels were compared manually to identify protein spots unique to particular strains.

MS protein sample preparation and LC-ESI–MS-MS. Coomassie-stained protein spots which were unique to the 2-DE gels of the *prfA*(*L140F*) mutant (NF-L1011), wild-type *prfA* (NF-L1041), or the Δ*prfA* mutant (NF-L1009) were cut out and subjected to liquid chromatography-electrospray ionization–tandem mass spectroscopy (LC-ESI–MS-MS) analysis at the Fred Hutchinson Cancer Research Center Proteomics Facility, Seattle, WA.

Coomassie-stained gel slice trypsin digestions were performed as described by Shevchenko et al. (58), omitting the alkylation and reduction steps. Following digestion, samples were desalted using a micro-C_{18} ZipTip (Millipore) and dried. Samples were resuspended in 7 μl of 0.1% trifluoroacetic acid and analyzed by LC-ESI–MS-MS using a Microtech Scientific UltraPlus2 high-pressure liquid chromatograph coupled in-line with a ThermoElectron LCQ mass spectrometer. The instrumental configuration is described by Gatlin et al. (24) and used a pulled 100-μm capillary as both the electrospray tip and the chromatographic column. The capillary was packed with 5-μm-particle-size, 100-Å-pore-size Magic C_{18} (Michrom Bioresources) packing material. High-pressure liquid chromatography solvents were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), and the separation gradient was 5% B to 40% B over 40 min. MS data were collected in a datum-dependent mode in which an MS scan was followed by MS-MS scans of the three most abundant ions from the preceding MS scan.

MS data were searched against four combined *L. monocytogenes* protein databases from TIGR (F2365, H7858, and F6854; http://www.tigr.org/tdb/listeria/) and the Pasteur Institute (EGD-e; http://genolist.pasteur.fr/ListiList/) by use of a software search algorithm (Institute for Systems Biology, Seattle, WA). Results were considered valid if at least two peptides identified to a protein and if the peptide matches had raw scores greater than 200 for +1 ions, 300 for +2 ions, and 300 for +3 ions, Z-scores greater than 4, and percent ions of greater than 15%. For further stringency, each protein was labeled as presented in Tables 4 and 5 if it was identified by MS in at least two out of three independent experiments. Proteins were given gene names based upon the sequenced EGD-e strain from the ListiList server (http://genolist.pasteur.fr/ListiList/).

Swimming motility assays. Bacterial swimming motility was measured as previously described (57, 72), with slight modifications. Briefly, swimming motility was evaluated on semisolid (0.3% [wt/vol] agar) BHI cam[5] plates stab inoculated from isolated colonies and incubated for 48 h at 37°C or at room temperature as indicated. Motility was quantified as the diameter of the swimming colony, measured at 24 and 48 h, minus the diameter of a nonmotile Δ*flaA L. monocytogenes* deletion mutant (NF-L1246), and wild-type (NF-L1041) motility was set to 100%. Results were obtained from triplicate samples of two independent experiments.

Plasmid insertional disruption mutagenesis of target genes in the *L. monocytogenes* chromosome. Five candidate genes and three control genes were selected for mutational analyses in *L. monocytogenes* by plasmid-based insertional disruption. Briefly, a 500-bp fragment of internal coding sequences of each candidate or control gene was chosen such that upon a single-crossover event a gene disruption was generated through targeted plasmid integration. Oligonucleotides (see Table S1 in the supplemental material) were designed for PCR amplification of internal coding sequences within the following genes: *actA*, *gap*, *rpoB*, lmo0135, *tcsA*, lmo1438, lmo2219, and lmo2417. The resulting PCR products were digested with XbaI and PstI and ligated into the temperature-sensitive plasmid vector pKSV7 (61) digested with the appropriate restriction enzymes.

As an integration control to determine the effect on *L. monocytogenes* fitness following plasmid integration, a pKSV7 construct originally intended for allelic exchange of lmo2219 was utilized (this construct results in a silent plasmid insertion and does not cause the insertional inactivation of any *L. monocytogenes* gene product). Briefly, oligonucleotides (see Table S1 in the supplemental material) were designed for PCR amplification of approximately 600 bp both upstream and downstream of the start and stop codons of lmo2219, respectively, and fused together via splicing by overlap extension PCR as previously described (31). The resulting PCR product was subcloned into pKSV7 following digestion with XbaI.

Plasmid constructs were subsequently electroporated into electrocompetent *L. monocytogenes* as previously described (30), incubated in BHI at 30°C for 2 h, plated onto BHI cam[10] plates, and incubated at 30°C for 2 days. Single colonies were picked and patched onto fresh BHI cam[10] plates and incubated overnight at 30°C. Colonies were then picked and inoculated into 3 ml BHI cam[10] media and passaged for 3 days with 1:1,000 dilutions at 40°C with vigorous shaking. The 3 days of passage at high temperature (40°C) prohibits the temperature-sensitive pKSV7 plasmid from replicating and selects for plasmid integration in the presence of antibiotic. For each mutant, plasmid integration was confirmed by PCR using one primer outside of the region of integration and another for the plasmid (see Table S1 in the supplemental material).

L2 plaque assays. Plaque assays were performed with murine L2 fibroblasts as described previously (63), with a multiplicity of infection of ~1:3. Plaque size was measured using a comparator (Finescale, Orange, CA). The average diameters

of at least 20 plaques from two independent experiments were determined for each strain, with the average wild-type (NF-L100) diameter being set to 100%.

Mouse intravenous infections. All animal procedures were IACUC approved. *L. monocytogenes* was grown in BHI or BHI cam^{10} for strains with plasmid insertions, with shaking at 37°C, to an OD_{600} of ~0.6. Bacterial cells were pelleted, washed in phosphate-buffered saline (PBS), and resuspended in PBS to reach a final concentration of approximately 4×10^4 bacteria/ml. Six to eight Swiss Webster mice (Charles River Laboratories, New York, NY) at 12 to 15 weeks old were infected via tail vein injection with 0.5 ml of bacterial culture (2×10^4 CFU). Three days postinfection, mice were sacrificed and livers and spleens were harvested. Organs were placed in 10 ml of 0.2% NP-40 (Nonidet P-40), tissues were homogenized using a Tissue Master 125 (Omni International, Marietta, GA), and 10-fold serial dilutions were plated onto BHI plates containing 200 μg/ml streptomycin. To ensure that plasmid integration was maintained throughout the mouse infection, at least 100 colonies/organ/mouse were replica plated onto BHI $cam^{7.5}$ plates.

An *actA* insertional disruption mutant was used as a negative control, and this mutant was largely cleared from infected mice: no bacteria were recovered from infected livers, and of those mice with bacteria in the spleen, 5 log fewer mutant than wild-type CFU were recovered (~10^3 versus ~10^8) (data not shown). Following replica plating onto BHI $cam^{7.5}$ plates to ensure plasmid maintenance during infection, ~0.1% of colonies tested lost the integrated plasmid (with the exception of the lmo0135i mutant (lmo0135 plasmid insertion mutant), for which 3.4% of recovered colonies lost the integrated plasmid), and 54.5% of the *actA* insertion mutants recovered lost the integrated plasmid (data not shown).

RESULTS

Direct comparison of levels of PrfA-dependent gene expression in *L. monocytogenes* strains containing different *prfA alleles.** A number of mutations within *prfA* that have been reported to lead to the constitutive expression of virulence genes in bacterial strains grown in broth culture (*prfA** mutations) have been identified over the past several years (52, 57, 67, 72). While some *prfA** mutants have been analyzed using functional assays (57, 67), a direct comparison of levels of virulence gene expression between multiple isogenic *prfA** mutants has not yet been reported. To identify the *prfA* mutation conferring the highest level of PrfA activation, four isogenic strains containing *prfA** mutations were chosen for direct comparison: *prfA*(*G145S*) (52), *prfA*(*E77K*), *prfA*(*G155S*), and *prfA*(*L140F*) (57, 67, 72) strains.

Whereas the *prfA*(*E77K*) and *prfA*(*G155S*) mutations can easily be introduced into the 10403S parent strain via allelic exchange (57), the *prfA*(*G145S*) and *prfA*(*L140F*) mutations appeared to confer subtle effects on bacterial growth that interfere with allelic exchange, as several attempts to reconstruct each of the mutations in wild-type strains proved unsuccessful (72; unpublished data). Therefore, to enable isogenic comparison of the four *prfA** alleles, each *prfA* mutation was introduced into a Δ*prfA L. monocytogenes* background by use of a pPL2-based integration vector (41) that contained the selected mutant *prfA* coding sequence along with all of the promoters that regulate *prfA* expression (Table 1). These *prfA*-pPL2 vector constructs were then integrated into the bacterial chromosome in single copy within the $tRNA^{Arg}$ gene without gene disruption (41). A similar construct containing wild-type *prfA*-pPL2 has previously been shown to functionally complement a *prfA* deletion strain (72). Drug selection (chloramphenicol) was maintained throughout the course of in vitro experiments to ensure retention of the integrated plasmid vectors.

To quantitate and compare the levels of PrfA activation in strains containing the mutant *prfA* alleles, the reporter gene *gus*, encoding GUS, was introduced as a transcriptional fusion downstream of *actA* within the *L. monocytogenes* chromosome (56). *actA* is a PrfA-dependent virulence gene that is normally expressed at low levels in bacteria grown outside of host cells but is highly expressed following the activation of PrfA, a transition that normally occurs in bacteria located within the host cell cytosol (56; Miner et al., submitted). All pPL2-complemented *prfA** strains exhibited similar growth characteristics in broth culture (Fig. 1C). As expected, strains that lacked functional PrfA (Δ*prfA*) (NF-L1009) or that contained a complementing wild-type copy of *prfA* on pPL2 (NF-L1041) exhibited very low levels of *actA* expression during growth in broth culture (Fig. 1A and B). However, the levels of *actA* expression for the various *prfA** mutant strains were found to be greatly increased in comparison. The *prfA*(*E77K*) mutant (NF-L1185) and the *prfA*(*G155S*) mutant (NF-L1186) exhibited significantly increased levels of *actA* expression (17-fold and 26-fold, respectively, at peak levels of expression) (Fig. 1B), while the *prfA*(*L140F*) mutant (NF-L1011) and the *prfA*(*G145S*) mutant (NF-L1226) were the most active PrfA* mutants tested (Fig. 1A), with peak expression levels that were 480-fold and 434-fold greater, respectively, than those of strains containing wild-type *prfA* (Table 2).

The *prfA** mutant strains were also compared by monitoring secreted LLO protein levels via Western analysis. Comparison of secreted protein fractions derived from the various *prfA** strains tested yielded LLO profiles that were consistent with mid-level activation of PrfA as conferred by the *prfA*(*E77K*) and *prfA*(*G155S*) mutant alleles, with higher levels of activation conferred by the *prfA*(*L140F*) and *prfA*(*G145S*) mutations (Fig. 1D). Strains containing the *prfA*(*L140F*) allele secreted the greatest amounts of LLO (an 18.9-fold increase in comparison to the amount for the wild type) (Table 2). The differences in magnitude of the relative severalfold increases for *actA* and *hly* expression as measured between the *prfA** and wild-type strains likely reflect the differential requirements of these promoters for PrfA activation. The *hly* promoter exhibits significant expression in broth culture in the absence of PrfA activation, whereas the *actA* promoter is expressed at low to undetectable levels under identical conditions (56). As the *prfA*(*L140F*) mutation appeared to confer the most substantial degree of PrfA activation as measured by both *hly* and *actA* expression, strains containing this mutation were chosen for the analyses of the effects of PrfA activation on *L. monocytogenes* secreted protein profiles.

Mutational activation of PrfA leads to altered patterns of protein secretion. Bacterial virulence factors are often either secreted or surface localized to promote interaction and engagement with host cell components. In order to identify proteins whose production and/or secretion was directly or indirectly dependent upon PrfA activation, supernatants were collected during the mid-log phase of bacterial culture growth, corresponding to the time at which PrfA* activity was determined to be maximal (Fig. 1), and proteins were collected via TCA precipitation and analyzed by 2-DE.

Strains lacking functional PrfA (Δ*prfA*) or containing wild-type *prfA* produced secreted protein profiles that appeared essentially identical (data not shown), consistent with the lack of PrfA activation during bacterial growth in broth culture (Fig. 1 and Table 2). The profile of secreted proteins derived from the mutationally activated PrfA* *prfA*(*L140F*) strain was

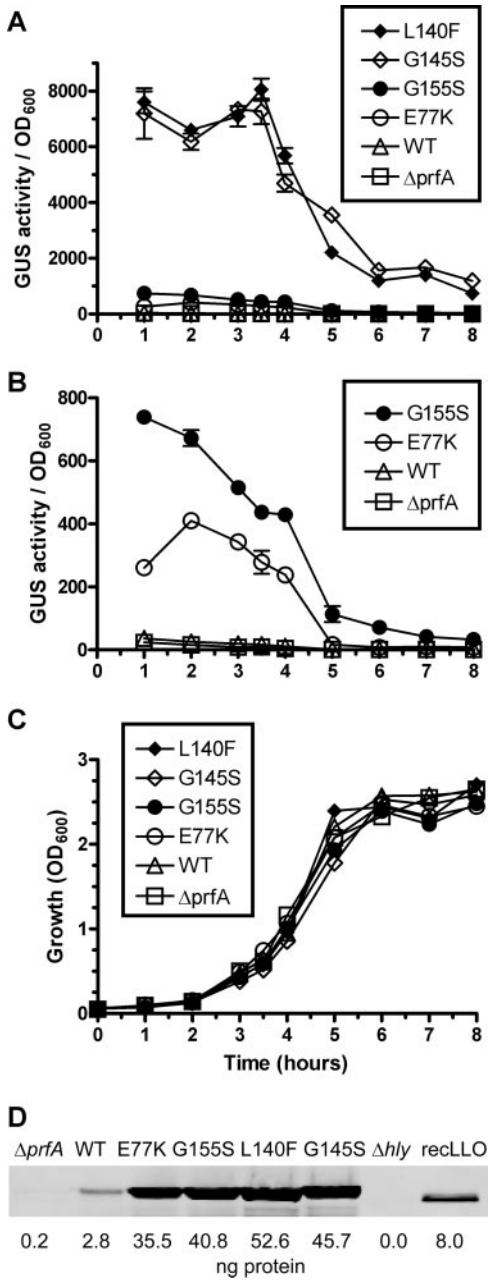

FIG. 1. *actA* expression levels and LLO secretion in isogenic *L. monocytogenes prfA** mutants grown in BHI broth culture at 37°C. Units of GUS activity were determined as described in Materials and Methods, following normalization for bacterial culture OD_{600}. Data shown are from duplicate samples and are representative of three separate experiments, expressed as averages ± standard deviations. (A) *actA* expression of wild-type *prfA* (WT), *prfA*(*L140F*), *prfA*(*G145S*), *prfA*(*G155S*), *prfA*(*E77K*), and Δ*prfA* isogenic strains. (B) *actA* expression as described for panel A, excluding the highly active *prfA*(*L140F*) and *prfA*(*G145S*) strains and expanded for clarity. (C) Growth curves of all strains in BHI broth culture at 37°C, as determined by OD_{600}. (D) Secreted proteins were TCA precipitated from bacterial supernatants during the mid-log phase of bacterial growth in BHI (OD_{600} of ~0.6). Proteins were separated using SDS-polyacrylamide gel electrophoresis, and LLO was detected by Western analysis using rabbit polyclonal antiserum directed against LLO followed by a fluorescently labeled goat anti-rabbit immunoglobulin G (IRDye 680) secondary antibody. Proteins were visualized using an Odyssey imager, and quantification was determined using Odyssey infrared imaging system application software, version 2.1. Strain genotypes are noted above each lane, and values below indicate nanograms of protein detected compared to 8 ng of recombinant LLO (recLLO). Data shown are representative of three separate experiments.

TABLE 2. *actA* expression as measured by GUS activity/OD_{600} for *prfA* mutant strains and LLO secretion as measured by Western analysis

Strain	Genotype or description	*actA*-GUS activity		LLO quantification	
		Avg value[a]	Fold Δ[b]	Secreted protein (ng)[c]	Fold Δ[b]
NF-L1041	Wild-type *prfA*	17 ± 1	1	2.8	1
NF-L1009	Δ*prfA*	10 ± 0	0.6	0.2	0.1
NF-L1185	*prfA*(*E77K*)	279 ± 26	17	35.5	12.7
NF-L1186	*prfA*(*G155S*)	437 ± 3	26	40.8	14.6
NF-L1226	*prfA*(*G145S*)	7,273 ± 456	434	45.7	16.3
NF-L1011	*prfA*(*L140F*)	8,055 ± 390	481	52.8	18.9

[a] Experimental mean units of GUS activity ± standard deviations. *actA*-GUS data are taken from duplicate samples and are representative of three independent experiments.

[b] Severalfold change in induction relative to values for strains containing wild-type *prfA*.

[c] Experimental quantification of secreted LLO protein based on Western blot comparisons with defined amounts of recombinant LLO. LLO secretion data are representative of three independent experiments.

strikingly different from wild-type or Δ*prfA*-derived profiles and showed enhanced expression of 22 polypeptide species as detected via Coomassie staining (Fig. 2). Three species were also notably absent in the PrfA*-derived supernatants but present in both the wild-type and Δ*prfA* supernatant fractions (Fig. 2). By comparison, *prfA*(*G145S*)-derived secreted protein profiles looked indistinguishable from those of *prfA*(*L140F*) strains, while the moderately activated PrfA* *prfA*(*E77K*) and *prfA*(*G155S*) mutants yielded similar yet less intense polypeptide patterns (data not shown), a result consistent with the high PrfA activation state of the *prfA*(*G145S*) mutant and the lower-level PrfA activation state of the *prfA*(*E77K*) and *prfA*(*G155S*) mutants (Fig. 1 and Table 2).

The differentially regulated polypeptide spots were excised from 2-DE gels and identified via LC-ESI–MS-MS. MS data were searched against all available *L. monocytogenes* protein databases, including two serotype 1/2a strains (EGD-e and F6854) and two serotype 4b strains (F2365 and H7858). From the 22 polypeptide spots up-regulated in the activated PrfA* strain, 15 proteins were identified (Table 3). Four of these, ActA, LLO, PlcB, and InlC, are encoded by genes that are known to be directly PrfA regulated via PrfA binding sites located within their promoter regions (47). Of the remaining 11 up-regulated proteins, only lmo2219 has been associated with PrfA regulation, as lmo2219 contains a potential PrfA binding site within its putative promoter region and was determined by microarray analyses to be up-regulated in a *prfA*(*G145S*) mutant (46). The 10 remaining up-regulated proteins, however, have not previously been associated with PrfA-dependent regulation, and the genes encoding these proteins all lack discernible PrfA binding sites within their predicted promoters or immediate upstream regions. The majority of these proteins have predicted functions that encompass four functional

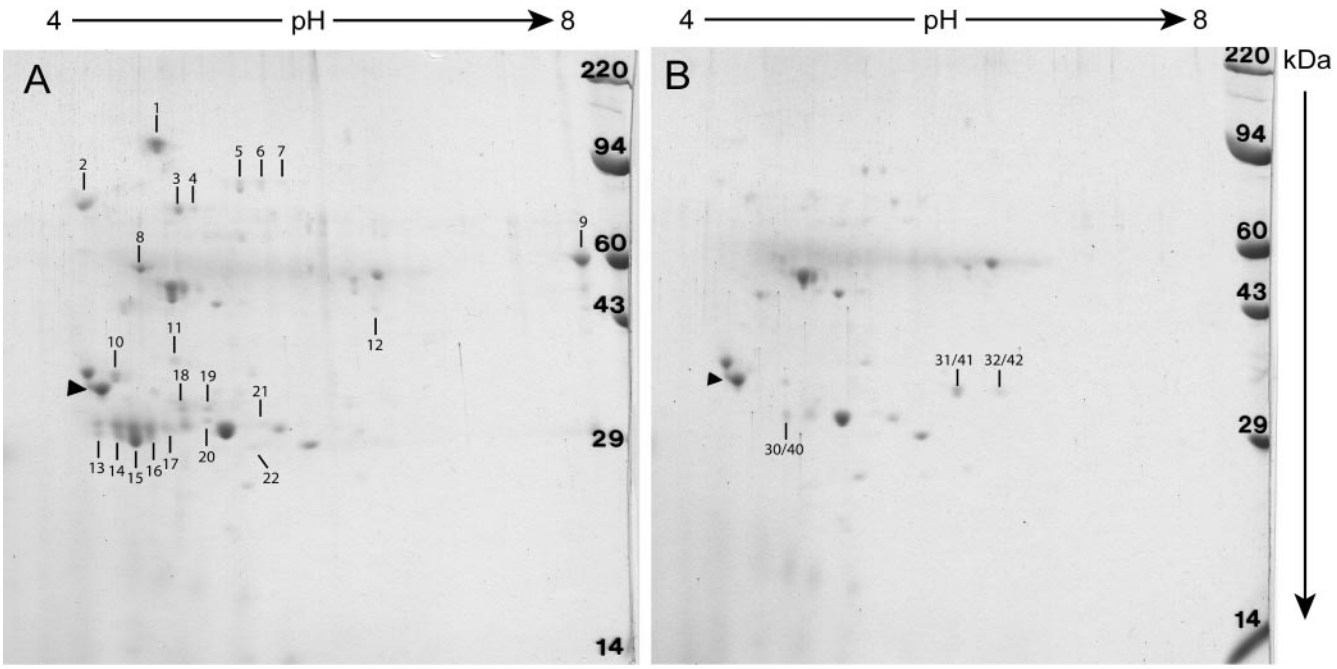

FIG. 2. 2-DE Coomassie-stained gels of secreted proteins isolated from isogenic *L. monocytogenes prfA* mutants. Secreted proteins were TCA precipitated from bacterial supernatants following approximately 3 h of bacterial growth in BHI (OD_{600} of ~0.6). Protein concentration was determined by BCA analysis, and equal amounts of protein were separated by 2-DE. Following electrophoresis, the gels were Coomassie stained and dried. Tropomyosin was included as a pI control (indicated by arrowhead), and a molecular size ladder (far right) was included. Polypeptide spots that were strain unique (indicated by vertical lines and numbers) were cut out and subjected to MS analysis. (A) *L. monocytogenes prfA*(*L140F*) mutant (NF-L1011). (B) *L. monocytogenes* wild-type *prfA* (NF-L1041).

classes: cell wall modification enzymes, components of protein secretion, ABC transporters, and unknown lipoproteins. The absence of apparent PrfA binding sites within the promoters and/or immediate upstream regions suggests that the PrfA*-dependent secretion of these gene products may occur via an indirect mechanism, such as the PrfA*-dependent expression of another cytosolic regulator, or alternatively via a secretion pathway that is dependent upon PrfA activation.

The secretion of QoxA and FlaA is reduced in *prfA strains, and the observed decrease in flagellar motility of *prfA** strains correlates with the lack of FlaA secretion.** Two proteins, QoxA and FlaA, were identified as present in the wild-type and Δ*prfA*-derived supernatants but absent in the supernatants derived from the *prfA*(*L140F*) strain (Table 4). The observed reduction in FlaA secretion was intriguing given that it has previously been shown that selected *prfA** mutants exhibited defects in swimming motility whereas strains lacking *prfA* exhibited increased motility in comparison to wild-type strains (57, 72). Consistent with these results, a direct comparison of each *prfA** mutant strain on soft agar indicated that PrfA* activity is inversely proportional to swimming motility (Fig. 3). Bacterial strains containing the most active *prfA** alleles [*prfA*(*L140F*) and *prfA*(*G145S*) strains] exhibited the greatest defects in swimming motility (60% ± 5.0% and 62% ± 4.3%, respectively, of the level of motility observed for strains containing wild-type *prfA*) (Fig. 3B). The reduced secretion of FlaA protein by *prfA** mutant strains establishes a functional link between PrfA activation and motility.

Identification of novel proteins secreted as a result of PrfA activation that contribute to *L. monocytogenes* virulence. Four of the 15 up-regulated proteins identified from the PrfA L140F secretome have well-established roles in *L. monocytogenes* virulence: ActA, LLO, PlcB, and InlC (66). The potential contributions of the remaining 11 identified proteins have not been well defined, although some of these proteins (Lmo0292 [HtrA], Lmo1438, Lmo2196 [OppA], Lmo2219, Lmo2691 [MurA, or NamA], and Lmo2754 [PBP5]) have been implicated in pathogenesis (4, 9, 10, 29, 42, 62, 70). To explore the pathogenic potential of additional proteins secreted from *L. monocytogenes* as a result of PrfA activation, the genes encoding five up-regulated proteins were selected for targeted disruption using the following criteria: (i) the existence of previously published data suggesting PrfA-dependent expression, (ii) similarity of the identified gene to virulence genes described for other bacterial pathogens, and/or (iii) lack of an established role in virulence in *L. monocytogenes*. At least one gene was chosen from each functional class, with the exception of the class of known virulence genes. Of the secreted proteins identified, the only novel gene product encoded by a gene with both a potential PrfA binding site in its promoter and existing evidence for PrfA-dependent gene expression was the putative protein secretion chaperone lmo2219 (46). Two of the three ABC transporters, encoded by lmo0135 and lmo2417, were targeted for insertional disruption, as the third (Lmo2196, also known as OppA) had previously been associated with virulence (4). One of the cell wall-modifying enzymes, Lmo1438, was selected, as well as the antigenic lipoprotein encoded by lmo1388 (also known as *tcsA*), based on its previous demonstration of expression within host cells and its capacity to stimulate T cells (54).

TABLE 3. Polypeptides unique or up-regulated in the PrfA L140F secretome

Functional class	Gene designation	Protein name	Protein description	Spot no.	Peptide match[a]	Coverage (%)[b]	Predicted pI	Predicted mass (kDa)	Observed pI	Observed mass (kDa)	Reference(s) or source for virulence contribution
Virulence	lmo0202	LLO	Listeriolysin O	9	124	72.0	8.2	58.7	7.7	60.0	38, 50
	lmo0204	ActA	Actin assembly-inducing protein	1	60	46.3	4.7	70.3	5.9	125.0	14, 40
				2	48	26.6			5.6	80.0	
				11	19	26.0			5.5	37.0	
	lmo0205	PlcB	Phospholipase C	13–17	7	23.5	8.4	33.3	5.8	30.0	65
	lmo1786	InlC	Internalin C	21	9	27.7	6.5	33.1	5.8	30.0	15, 21
ABC transporter	lmo0135		Similar to oligopeptide ABC transport system substrate-binding proteins	8	40	53.6	4.8	58.3	5.9	58.0	This study
	lmo2196	OppA	Similar to pheromone ABC transporter (binding protein)	3	30	49.6	5.1	62.6	6.0	80.0	4
	lmo2417		Conserved lipoprotein (putative ABC transporter binding protein)	13–17	4	15.6	5.1	30.7	5.8	30.0	
Cell wall modifying	lmo1438		Similar to penicillin-binding protein	5	10	17.5	8.3	79.9	6.2	85.5	29
				6	31	43.0			6.3	85.5	
				7	36	51.5			5.8	83.0	
	lmo2522		Similar to hypothetical cell wall binding protein from *B. subtilis*	13–17	6	15.9	5.8	29.1	5.8	30.0	
				17	5	23.1			6.5	14.0	
				20	33	63.9			5.7	30.5	
	lmo2691	MurA or NamA	Similar to autolysin, *N*-acetylmuramidase	3	2	7.1	10.3	63.6	6.0	80.0	9, 42
				3 and 4	7	14.7			6.0	78.0	
	lmo2754	PBP5	Similar to D-alanyl-D-alanine carboxypeptidase (penicillin-binding protein 5)	12	20	42.5	7.6	48.1	6.8	45.0	29
Antigenic lipoprotein	lmo1388	TcsA	$CD4^+$ T-cell-stimulating (surface) antigen, lipoprotein	10	18	59.4	4.8	38.4	5.3	35.0	This study
	lmo2637		Conserved lipoprotein (putative)	18	22	36.1	6.6	32.7	6.0	33.0	
				19	2	10.7			5.7	32.0	
Secretion	lmo0292	HtrA	Similar to heat shock protein HtrA serine protease	2	8	21.6	4.3	52.8	5.6	80.0	62, 70
	lmo2219	PrsA2	Similar to posttranslocation molecular chaperone (protein export protein) (PrsA from *B. subtilis*)	19	13	39.2	5.6	32.7	5.7	32.0	10, this study
				20	17	43.7			5.6	31.0	

[a] Number of tryptic peptides detected that could be matched to each protein.
[b] Amino acid coverage calculated from the whole protein.

The targeted genes were disrupted through plasmid integration using the temperature-sensitive pKSV7 plasmid and selection for single-crossover events. Plasmid vector insertion controls including pKSV7 with internal fragments of the essential housekeeping genes *gap* or *rpoB* or empty pKSV7 vector failed to produce any chloramphenicol-resistant colonies at 40°C, confirming the site specificity of plasmid integration and the essential roles of *gap* and *rpoB* in *L. monocytogenes* physiology (data not shown). Of the five genes selected for plasmid disruption, the strain containing the plasmid insertion targeted

TABLE 4. Polypeptides absent or down-regulated in the PrfA L140F secretome

Functional class	Gene designation	Protein name	Protein description	Spot no.	Peptide match[a]	Coverage (%)[b]	Predicted pI	Predicted mass (kDa)	Observed pI	Observed mass (kDa)	References for virulence contribution
Motility	lmo0690	FlaA	Flagellin	40	30	63.1	4.7	30.4	5.9	32.5	16, 49
Energetics	lmo0013	QoxA	AA3-600 quinol oxidase subunit II	41	28	45.1	6.4	41.6	7.0	34.5	
				42	7	20.7			6.9	34.6	

[a] Number of tryptic peptides detected that could be matched to each protein.
[b] Amino acid coverage calculated from the whole protein.

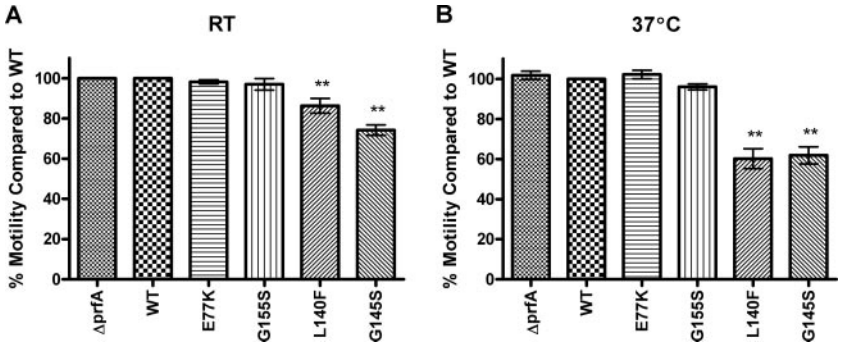

FIG. 3. Swimming motility of isogenic *L. monocytogenes prfA* mutants in semisolid media. Single colonies were stab inoculated into BHI 0.3% agar plates and incubated at room temperature or at 37°C. Swimming motility was calculated as the diameter of the halo as measured at 24 and 48 h minus the diameter of the nonmotile Δ*flaA L. monocytogenes* deletion mutant (NF-L1246). Motility data are representative of triplicate samples obtained from two separate experiments and are expressed as averages ± standard errors of the means. For statistical analysis, all strains were compared against pPL2 *prfA*-WT (NF-L1041) by use of the Dunnett test and were calculated using GraphPad InStat, version 3.06 (San Diego, CA). Swimming motility at (A) room temperature (RT) or (B) 37°C, with wild-type (WT) motility set at 100%. **, $P < 0.01$.

for lmo1438 failed to grow under selective conditions (data not shown); this result strongly suggests that lmo1438 is an essential gene in *L. monocytogenes* strain 10403S. The remaining four plasmid insertional disruption mutants all exhibited similar growth rates in broth culture (data not shown).

The ability of *L. monocytogenes* to enter host cells, escape from vacuoles, replicate within the cytosol, and spread to adjacent cells can be assessed based on the ability of bacteria to form zones of clearing or plaques in tissue culture monolayers (63). The number of plaques formed and the size of the plaques can be used as indications of bacterial invasion and cell-to-cell spread, respectively. To compare levels of intracellular growth of the targeted gene disruption mutants in tissue culture cells, murine L2 monolayers were infected with each mutant strain and plaques were measured 3 days postinfection. All of the gene disruption mutants formed plaques of approximately the same size and frequency as wild-type *L. monocytogenes*, with the exception of the lmo2219 mutant, which was observed to yield slightly smaller plaques (86% that of the wild type) (Table 5), suggestive of a modest intracellular growth defect. In comparison, a mutant containing an insertional disruption of *actA* failed to produce any plaques, resulting from the inability of this strain to spread to adjacent cells (Table 5) (6).

TABLE 5. Comparison of plaque formations by the *L. monocytogenes* gene disruption mutants

Strain	Associated gene designation or description	Plaque size (% of wild type)[a]
NF-L100	Wild type	100 ± 9.9
NF-L1239	*actA*i	0
NF-L1239	lmo0135i	100.5 ± 7.6
NF-L1239	*tcsA*i	95.4 ± 10.6
NF-L1239	lmo2219i	86.3 ± 5.6***
NF-L1239	lmo2417i	99.6 ± 6.0

[a] Plaque sizes (±standard deviations) of L2 fibroblasts infected with insertion mutants. The mean plaque size of the wild-type strain was defined as 100%. The data shown here are representative of results of two independent experiments. For statistical analysis, all strains were compared against the wild-type strain NF-L100 by use of unpaired *t* tests with two tails and were calculated using GraphPad InStat, version 3.06 (San Diego, CA). ***, $P < 0.001$.

To address the potential contributions of the selected genes to animal infection, the gene disruption mutants were examined for the ability to replicate within the livers and spleens of infected mice. Twelve- to 15-week-old Swiss Webster mice were injected intravenously, and the livers and spleens were harvested at 72 h postinfection to assess bacterial load. Mutants were compared against two different reference strains: DP-L3903 (a 10403S derivative containing a silent Tn*917* insertion that is fully virulent in mouse intravenous infections [1]) and NF-L1245, which contains a silent pKSV7 insertion within the *L. monocytogenes* genome. Both DP-L3903 and NF-L1245 were fully virulent, based on their ability to replicate within the livers and spleens of infected animals (Fig. 4). In contrast, three of the four gene disruption mutants showed significant defects in virulence, based on the reduced bacterial numbers recovered from both liver and spleen (Fig. 4). The *tcsA* insertion mutant (lmo1388) resulted in more than a 10-fold decrease in bacterial CFU recovered from the liver and spleen in comparison to wild-type strains, whereas the lmo0135 and lmo2219 gene disruption mutants were reduced in bacterial numbers by 2 log or more. The lmo2417 gene disruption mutant showed no significant defect. The lmo1388, lmo0135, and lmo2219 gene products therefore are not required for *L. monocytogenes* growth in broth culture but are required for full bacterial virulence in infected mice.

DISCUSSION

PrfA activation is believed to be critical for the ability of *L. monocytogenes* to transition from life as an environmental organism to life within a mammalian host (28). Activation occurs following entry of *L. monocytogenes* into the cytosol of infected host cells (Miner et al., submitted) and leads to the expression of gene products that promote bacterial replication and spread to adjacent cells. Although significant progress towards identifying gene products directly regulated by PrfA has been made through the use of genome analysis and microarrays (26, 46), the full impact of PrfA activation on *L. monocytogenes* protein expression and bacterial cell physiology has not yet been examined. By using the mutational activation of PrfA as a tool for

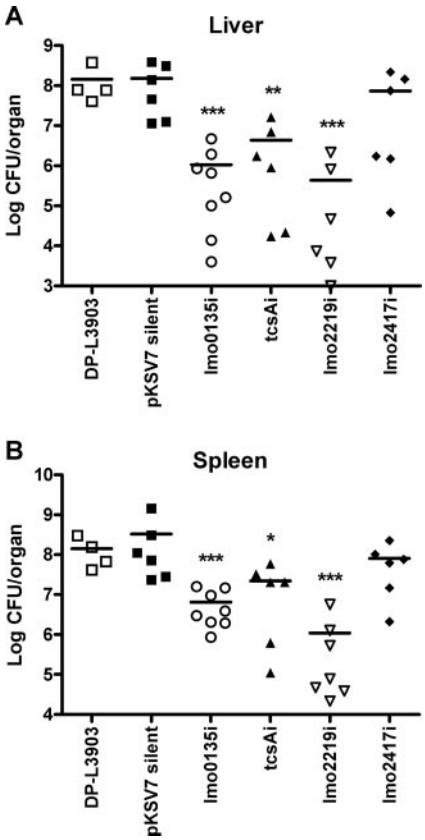

FIG. 4. Bacterial growth in mouse livers and spleens following intravenous infection with *L. monocytogenes* gene disruption mutants. *L. monocytogenes* mutant strains were grown to mid-log phase in BHI broth, the bacteria were washed with PBS, and 2×10^4 CFU were injected via tail vein into six to eight 12- to 15-week-old Swiss Webster mice. Mice were euthanized at 3 days postinfection, organs were harvested and homogenized, and aliquots of tissue suspensions were plated on selective media for determination of bacterial CFU. Data are presented as scatter plots of CFU, with mean values indicated by horizontal bars. For statistical analysis, all strains were compared against the silent pKSV7 integration mutant NF-L1245 by use of unpaired *t* tests with two tails of transformed data, which were calculated using GraphPad InStat, version 3.06 (San Diego, CA). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Results were nearly identical when candidates were compared against DP-L3903, with the exception that the *tcsA*i spleen data were no longer significant ($P = 0.0656$). i, the pKSV7 plasmid insertion mutant.

elucidating changes in protein production and secretion, this study has identified 17 proteins whose secretion is influenced by PrfA activation and has further implicated three novel proteins in *L. monocytogenes* virulence.

Current PrfA* mutants can be classified into at least two categories based on the expression of PrfA-dependent virulence genes in *L. monocytogenes* broth cultures: moderately active PrfA* mutants (PrfA E77K and PrfA G155S mutants) and highly active PrfA* mutants (PrfA G145S and PrfA L140F mutants). The existence of two distinguishable PrfA* mutant classes could indicate that PrfA is capable of undergoing a progressive conformational shift from an inactive species into a fully active state, with the moderately active class of mutants representing an intermediate step within that progression. Alternatively, the mid-level-activation mutants may be reflective of an artificial conformation imposed on PrfA structure by each of the two mutations. Structural comparisons between wild-type PrfA and PrfA G145S mutant proteins by Eiting et al. (19) have revealed a conformational difference that exists between the two proteins such that the helix-turn-helix region responsible for DNA binding appears more accessible in the PrfA G145S mutant. The ability of the *prfA*(*E77K*) and *prfA*(*G155S*) mutant strains to remain fully virulent (or even hypervirulent) in animal models of infection strongly suggests that the moderately active PrfA* mutants retain the ability to become fully activated within host cells (53, 57).

The proteins identified in this study as differentially secreted in the presence of one of the most active *prfA** alleles, *prfA*(*L140F*), fall into several functional categories, including ABC transport components, cell wall modification enzymes, antigenic lipoproteins, secretion machinery components, metabolic enzymes, and components necessary for swimming motility. Recent studies have implicated some of these proteins in bacterial virulence, whereas the roles of others remain to be defined. For example, ABC transporters couple ATP hydrolysis with transport of small molecules across bacterial membranes, and oligopeptide ABC transporters have been shown to be involved not only in nutrient acquisition but also in protein secretion and quorum sensing in gram-positive bacteria (13, 27). Three ABC transport components were identified in the *prfA*(*L140F*) mutant secretome: Lmo0135, Lmo2196 (OppA), and Lmo2417. OppA has previously been shown to mediate transport of oligopeptides and to contribute to intracellular growth and survival of bacteria in bone marrow-derived macrophages and in the livers and spleens of infected mice (4). Based on the reduced numbers of the lmo0135 insertion mutant CFU recovered from the livers and spleens of infected mice (Fig. 4), the lmo0135 gene product contributes to *L. monocytogenes* pathogenesis in animals, perhaps by providing transport of a nutritional component required for growth within the host but not required for bacterial growth in vitro.

Interestingly, the largest class of secreted proteins identified from the *prfA*(*L140F*) strain consisted of gene products predicted to modify the structure and/or integrity of the bacterial cell wall (Lmo1438, Lmo2522, Lmo2691, and Lmo2754). The two penicillin-binding proteins identified, Lmo1438 and Lmo2754 (PBP5), have recently been shown to contribute to *L. monocytogenes* virulence, as gene disruption mutants injected intraperitoneally into mice yielded 2-log and 1-log reductions, respectively, in bacterial CFU recovered from the spleen (29). Strain differences may account for the inability to generate an insertional disruption mutant in lmo1438 in strain 10403S, whereas Guinane et al. (29) were successful in disrupting lmo1438 in the EGD-e background by using a similar method.

Additional proteins found to be up-regulated in *prfA*(*L140F*) supernatants included Lmo1388 (TcsA), Lmo2637, Lmo2219, and Lmo0292 (HtrA). TcsA was first identified in a screen for *L. monocytogenes* $CD4^+$ T-cell-stimulating antigens (54), and it shares sequence similarity with the Bmp (*b*asic *m*embrane *p*rotein) family of proteins found in a variety of bacteria, including various *Bacillus* and *Streptococcus* species, as well as pathogenic spirochetes, including *Treponema pallidum* and *Borrelia burgdorferi*, in which the Bmp proteins are highly antigenic outer membrane proteins of unknown function (7). In *L. monocytogenes*, TcsA is located at the 5′ end of a series of genes which appear to

comprise a sugar ABC transport system; thus, TcsA could potentially be involved in the bacterial transport of carbohydrates within the host. HtrA (also known as DegP) is a member of a highly conserved serine protease family and is thought to degrade misfolded proteins and potentially function as a chaperone. HtrA is known to contribute to virulence in several gram-negative and gram-positive bacterial pathogens, such as *Salmonella enterica* serovar Typhimurium (34), *Pseudomonas aeruginosa* (5), *Streptococcus pneumoniae* (32), *Staphylococcus aureus* (51), and *Streptococcus pyogenes* (35), presumably by assisting in the secretion and maturation of virulence factors (44). In *L. monocytogenes*, HtrA has been shown to be essential not only for bacterial resistance to high salt (71) and oxidative stress (70) but also for bacterial virulence in mice (62, 70).

A second potential secretion chaperone identified as a result of PrfA activation was Lmo2219. lmo2219 contains a putative PrfA binding site in its promoter region and has previously been associated with PrfA-dependent regulation by transcriptome analysis (46). Lmo2219 has significant homology to PrsA from *Bacillus subtilis* (45% identity, 63% similarity) and other peptidyl-prolyl isomerases and is thought to act as a chaperone for secreted proteins. A recent study identified the expression of lmo2219 as up-regulated inside of host cells, and a Δlmo2219 strain exhibited a defect in bacterial growth and spread within fibroblast monolayers as well as decreased growth in bone marrow-derived macrophages (10). In this study, the lmo2219 gene disruption mutant was found to exhibit the largest virulence defect of all mutants tested (Fig. 4). Two *prsA*-like genes with peptidyl-prolyl isomerase domains are present in the *L. monocytogenes* genome, lmo1444 and lmo2219, and we therefore propose to name them *prsA1* and *prsA2*, respectively, following the annotation given by TIGR in their *L. monocytogenes* sequence databases (http://www.tigr.org/tdb/listeria/). In *B. subtilis*, *prsA* has been shown to be an essential gene (68). The presence of two *prsA*-like genes in *L. monocytogenes* and the ability to inactivate lmo2219 suggest the existence of some functional redundancy or perhaps a division of substrate specificity between the two chaperones whereby the lmo2219 gene product may contribute to the folding of extracellular virulence factors.

Finally, two proteins were identified as down-regulated or absent in the *prfA*(*L140F*) secretome: Lmo0013 (QoxA, quinol oxidase subunit II) and Lmo0690 (FlaA). In *B. subtilis*, QoxA, together with QoxB, QoxC, and QoxD, forms an oligomeric enzymatic complex on the cell membrane which catalyzes the transfer of electrons from quinol onto molecular oxygen to form water in the terminal step of the respiratory chain (55). However, terminal oxidases may take on alternate roles as electron scavengers in order to detoxify the intracellular environment triggered by the antimicrobial response during infection; such has been proposed with cytochrome *bd* in both *Shigella flexneri* (69) and *Brucella abortus* (20). FlaA, the major subunit of flagella, was also found to be down-regulated or absent in the *prfA*(*L140F*) secretome, a finding which correlates with the decrease in swimming motility observed for *prfA** mutants (Fig. 3). The decrease in secreted FlaA suggests that activated PrfA may either down-regulate *flaA* gene expression or interfere with FlaA assembly. Evidence for PrfA-dependent regulation of *L. monocytogenes* motility gene expression exists: *motA* transcripts (encoding a component of the flagellar motor in the plasma membrane) appear to be repressed by PrfA (*motA* expression is twofold greater in a Δ*prfA* strain at 37°C) (45), and *flaA* transcripts were reduced in the presence of the *prfA*(*G145S*) mutation at 20°C (52).

Very recently, Baumgartner et al. (2) undertook a proteomic analysis of lipoproteins in *L. monocytogenes* by deleting the prolipoprotein diacylglyceryl transferase (Lgt), which led to a release of lipoproteins into the culture supernatant. Twenty-six of the 68 predicted lipoproteins were identified in the extracellular proteome of the Δ*lgt* strain, including most of the lipoproteins identified in this study. This suggests that PrfA activation results in the overexpression and/or hypersecretion of lipoproteins such that a significant number are shed into the extracellular environment. Alternatively, PrfA activation may result in an alteration of the bacterial cell membrane or cell surface that results in the release of lipoproteins; changes in *L. monocytogenes prfA*(*L140F*) mutant surface properties have been reported previously (72). Baumgartner et al. (2) further found that overexpression of wild-type *prfA* from a multicopy plasmid in the Δ*lgt* strain resulted in the up-regulation of six proteins, four of which were identified in this study (ActA, InlC, Lmo2219 [PrsA2], and Lmo2196 [OppA]) and two which were not (InlA and Lmo0366). The difference in the numbers of proteins identified is likely reflective of both the higher activation state of the *prfA*(*L140F*) allele used here (versus the overexpression of nonactivated wild-type protein) and the presence of functional Lgt.

This study demonstrates that upon PrfA activation the *L. monocytogenes* secretome is highly enriched for proteins that contribute to virulence. Although targeted gene disruption via plasmid insertion does not exclude the possibility of polar effects of the insertions on downstream gene expression, the disruption mutants are still clearly useful for identifying regions of the *L. monocytogenes* chromosome that contribute to bacterial pathogenesis. Since several of the gene products identified were not described as up-regulated in a transcriptome analysis of the mutationally activated PrfA G145S strain (46) and lack obvious PrfA binding sites within their promoter regions, it appears that a PrfA-dependent product or pathway (rather than direct activation by PrfA) is responsible for their altered patterns of expression and/or secretion. These studies highlight the significant and broad-range influences of PrfA activation on *L. monocytogenes* protein expression profiles and on bacterial virulence.

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